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Immunological studies on treponemal cellular antigens *I. Serological changes and resistance to infection in rabbits immunized with treponemal nucleic acids*

N. N. IZZAT AND E. YAZDI
Departments of Dermatology and Syphilology, and Pathology, Baylor College of Medicine, Houston, Texas 77025, *and Pathology Research Laboratories, Veterans Administration Hospital, Houston, Texas* 77031, *U.S.A.*

Immunochemical as well as serological studies by various investigators (Cannefax and Garson, 1959; Christiansen, 1964; D'Alessandro and Dardanoni, 1952; Izzat, Dacres, Knox, and Wende, 1970) have shown that avirulent strains of *Treponema pallidum*, like other micro-organisms, possess a complex antigenic structure. These complexes include protein, polysaccharide, and lipid components. To our knowledge, treponemal nucleic acid antigens have never been investigated. We have attempted to free these antigens and test their immunological activity in rabbits.

Material and methods

The avirulent Nichols strain of *T. pallidum* was cultivated in non-synthetic medium (Izzat, Knox, Dacres, and Smith, 1971a; Izzat, Knox, and Wende, 1971b). Freshly isolated cells were used for nucleic acid preparation.

Adult New Zealand male rabbits, weighing five to six pounds, with non-reactive VDRL tests and no evidence of infection with *T. cuniculi*, were used throughout the study. They were housed in individual cages in a specially designed room that kept the environmental temperature at precisely 70°F.

Preparation of treponemal nucleic acids Cultures of avirulent *T. pallidum* (Nichols) were subjected to continuous flow centrifugation using a Servall RC-2 centrifuge at 37,000 G. The cells were compacted in a 20-ml. vial and used immediately. 50 g. wet weight of the cell preparation were washed in saline (0·15 M) and sonicated (Izzat and others, 1971a,b). RNA was extracted by the phenol-sodium lauryl sulphate method (Steele and Busch, 1967). DNA was extracted according to the method of Kay, Simmons, and Dounce (1952). The DNA and RNA preparations were stored at —20°C. until use.

Treponemal nucleic acid determination Equal volumes of 1·0 N ice-cold perchloric acid were added to the treponemal suspensions in culture media and the specimens centrifuged. The pellets were washed once more with ice-cold 0·5 N perchloric acid, stored in acetone overnight, and washed sequentially with ethanol, ethanol-ether, and ether, and then air-dried according to the Schmidt-Thannhauser procedure (Leslie, 1955). Lipid-free, air-dried samples were hydrolysed with 0·3 N KOH for 20 to 24 hrs at 37°C. The hydrolysates were acidified with cold perchloric acid (PCA) and centrifuged. The precipitates were washed with cold 0·5N PCA, and the supernatant pooled and used for RNA determination by the Orcinol method (Drury, 1948). The precipitates were hydrolysed with 0·5N PCA at 100°C. for 20 minutes. The hydrolysates were centrifuged, the precipitate washed with 0·5 N PCA, and the supernatants pooled and used for DNA determination according to the method of Burton (1956). Yeast RNA and calf thymus DNA were used as standards. Treponemal RNA and DNA were expressed as μg. per mg. lipid-free dry weight of samples, as well as pg. per cell.

Immunization procedure For immunization, the RNA and DNA preparations were centrifuged and suspended in Tris-HCl buffer (pH 7·2). This buffer contains 0·025 M KCl and 0·005 M $MgCl_2$. In this buffer, the RNA material produced a clear solution. The DNA-Tris suspension was placed on a mechanical stirrer at 4°C. Stirring continued for 2 days with the addition of sterile NaCl crystals to a final concentration of 0·14 M. Aliquots of DNA and RNA solutions were used for determination of RNA and DNA, according to Drury (1948) and Burton (1956) respectively. After determining the amounts of RNA and DNA, both the RNA and DNA preparations were incorporated into Freund's complete adjuvant by the method of Youmans and Youmans (1967).

Two groups of animals were injected with the above preparations in one of two ways. The first group of eight rabbits was immunized intravenously over a period of 9 weeks; four rabbits received biweekly 25 μg. DNA per injection and the remaining four rabbits received biweekly 250 μg. RNA per injection. Two rabbits were added to this group to serve as challenge controls. The second group of fourteen rabbits was immunized subcutaneously over a period of 12 weeks; six rabbits received the DNA-adjuvant emulsion, each receiving 50 μg. DNA on a weekly basis. The remaining eight rabbits received the RNA-adjuvant emulsions, each receiving 500 μg. RNA weekly. An additional two rabbits were added to this group to serve as challenge controls.

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Infectivity test After the completion of the immunization schedule, all the experimental rabbits and the four controls were challenged intradermally with 100 virulent *T. pallidum* suspended in 0·1 ml. of 50 per cent. rabbit serum in saline. Each rabbit received four injections at four sites of the skin on the lower back. Rabbits were observed daily for the development of chancres. All lesions were subjected to darkfield examination.

Serological tests Tests for syphilis (VDRL and FTA-ABS) were made 10 weeks before challenge and 6 weeks after challenge. The Houston City Health Department Laboratory, Houston, Texas, performed these tests.

Results

As nucleic acid formation increased by augmenting cell division, it was important to demonstrate in our culture system when the treponemal culture had a maximum level of nucleic acid. Table I and the Figure show that RNA/DNA ratios were sequentially related to the treponemal growth cycle. The maximum level of nucleic acid was achieved by Day 6, which was followed with a plateau until Day 9. Consequently, 9-day cultures were selected for the preparation of rich DNA and RNA immunizing material.

TABLE I *Nucleic acids content of avirulent* Treponema pallidum *Nichols strain**

Day of incubation	*Number of cells* $\times 10^6$*/ml.*	*RNA (pg./cell)*	*DNA (pg./cell)*	*Ratio RNA/DNA*
0	22·00	6·53	2·18	2·9
1	18·60	4·11	1·14	3·6
2	19·42	2·04	0·94	2·17
3	37·30	3·61	0·66	5·47
4		5·41	0·46	11·76
5		4·43	0·35	12·65
6	55·40	5·40	0·39	13·84
7	63·16	5·98	0·44	13·59
8	82·16	4·80	0·35	13·71
9	86·00	4·31	0·35	12·31

*Average of four experiments; two or three determinations per experiment
pg = picogram

Administration of the above RNA and DNA preparations intravenously did not cause any change in the reactivity of the VDRL and FTA-ABS tests (Table II), with the exception of one rabbit (No. 1390) which showed positive reactivity to the FTA-ABS test after 10 weeks of immunization.

Lack of reactivity to the FTA-ABS test was also demonstrated in animals that received RNA-adjuvant mixture subcutaneously and only four rabbits showed changes in the VDRL reactivity (Table III B, overleaf). This is in contrast to animals that received DNA-adjuvant mixture subcutaneously, in which both the VDRL and FTA-ABS tests became reactive (Table III A, overleaf).

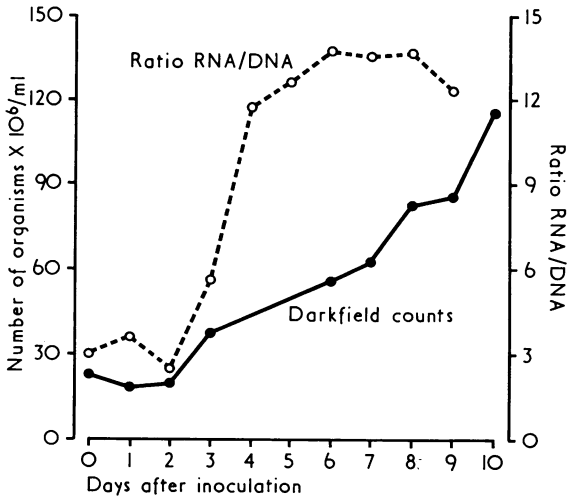

FIGURE *Relation of RNA/DNA ratios to treponemal growth cycle*

TABLE II *Serological changes in rabbits immunized intravenously with nucleic acids*

Series	*Rabbit no.*	*Serological test*	*Pre-immunization*	*Post-immunization 10 wks*	*Post-challenge[a] 6 wks*
A DNA (450 μg/. rabbit)	1386	VDRL	NR	NR	R 1:2
		FTA-ABS	NR	NR	R
	1387	VDRL	NR	NR	R 1:8
		FTA-ABS	NR	NR	R
	1388	VDRL	NR	NR	R 1:2
		FTA-ABS	NR	NR	R
	1389	VDRL	NR	NR	R 1:4
		FTA-ABS	NR	NR	R
B RNA (4,500 μg/. rabbit)	1390	VDRL	NR	NR	R 1:16
		FTA-ABS	NR	R	R
	1391	VDRL	NR	WR	R 1:2
		FTA-ABS	NR	NR	R
	1392	VDRL	NR	NR	R 1:2
		FTA-ABS	NR	NR	R
	1393	VDRL	NR	NR	R 1:16
		FTA-ABS	NR	NR	R
C Controls	1394	VDRL	NR		WR
		FTA-ABS	NR		R
	1395	VDRL	NR		WR
		FTA-ABS	NR		R

Abbreviations: NR nonreactive; R reactive; WR weakly reactive
[a]All rabbits had darkfield positive lesions

TABLE III *Serological changes in rabbits immunized subcutaneously with nucleic acids*

Series	*Rabbit No.*	*Serological test*	*Pre-immunization*	*Post-immunization (10 wks)*	*Post-challenge[a] (6 wks)*
A DNA (600μg./rabbit)	1396	VDRL	NR	WR	R 1:4
		FTA-ABS	NR	R	R
	1397	VDRL	NR	NR	R 1:8
		FTA-ABS	NR	R	R
	1398	VDRL	NR	WR	R 1:4
		FTA-ABS	NR	R	R
	1399	VDRL	NR	WR	R 1:2
		FTA-ABS	NR	R	R
	1400	VDRL	NR	R:UND	R 1:8
		FTA-ABS	NR	NR	R
	1401	VDRL	NR	R 1:2	WR
		FTA-ABS	NR	R	R
B RNA (6000 μg./rabbit)	1402	VDRL	NR	NR	R 1:16
		FTA-ABS	NR	NR	R
	1403	VDRL	NR	WR	R 1:2
		FTA-ABS	NR	NR	R
	1404	VDRL	NR	NR	R 1:16
		FTA-ABS	NR	NR	R
	1405	VDRL	NR	NR	R 1:4
		FTA-ABS	NR	NR	R
	1406	VDRL	NR	NR	R 1:64
		FTA-ABS	NR	NR	R
	1407	VDRL	NR	WR	R 1:2
		FTA-ABS	NR	NR	R
	1408	VDRL	NR	WR	R 1:8
		FTA-ABS	NR	NR	R
	1409	VDRL	NR	WR	WR
		FTA-ABS	NR	NR	R
C Controls	1410	VDRL	NR		R 1:2
		FTA-ABS	NR		R
	1411	VDRL	NR		R 1:4
		FTA-ABS	NR		R

Abbreviations: NR nonreactive; R reactive; WR weakly reactive
[a]All rabbits had darkfield positive lesions

After challenge with virulent *T. pallidum*, darkfield positive lesions appeared at the same time (20 to 23 days) in both immunized and control rabbits. Serological changes were observed in all rabbits 6 weeks after challenge.

Discussion

The results obtained in the present study clearly indicate the existence of an active biosynthesis of nucleic acid throughout the growth cycle. Also, the endogenous nucleic acid biosynthesis was directly related to cellular growth, analogous to that in bacterial and mammalian cells. Consequently, the aged treponemal cultures (6 to 9 days) were chosen to yield the highest amount of nucleic acid for antigenic testing.

Repeated intravenous administration of treponemal RNA and DNA in rabbits did not cause the production of antibodies that could be detected by VDRL and FTA-ABS tests. It is very probable that the intravenous route of administration resulted in a direct exposure of RNA and DNA antigens to the action of nucleases *in vivo* or that the injected antigens were utilized by the rabbit defence system as a non-specific stimulator without specific concurrent antibody production. Studies by Jaroslow and Taliaferro (1956), Taliaferro and Jaroslow (1960), Askonas and Rhodes (1965), and Mowbray and Scholand (1966) support the latter view.

Repeated subcutaneous injections of DNA-adjuvant resulted in the production of syphilitic antibodies that could be detected by VDRL and FTA-ABS tests. Our results also showed that RNA injections were able to induce VDRL reactivity in 50 per cent. of the animals tested but not FTA-ABS reactivity. The question of how the DNA preparations were able to induce both syphilitic antibodies can only be speculated upon. It is probable that this type of DNA antibody inducement might be related to an adjuvant action of nucleic acids (Merritt and Johnson, 1965) on a small amount of contaminating treponemal antigen. Conceivably, DNA, as well as Freund's adjuvant, might activate the reticuloendothelial system, so that the smaller amount of contaminating antigen was sufficient to induce the circulating syphilitic antibodies.

Induction of serological responses by the DNA preparation and those developed after challenge in the remaining animals were of no significant value in protection. This finding is in agreement with that of Magnuson, Thompson, and McLeod (1951), Miller (1965), and Izzat and others (1970), that syphilitic antibody production is not related to the animal's resistance to infection.

Summary

The growth of avirulent *Treponema pallidum in vitro* is directly related to nucleic acid biosynthesis. Rabbits injected subcutaneously with a DNA-adjuvant mixture developed VDRL and FTA-ABS antibodies. These seroreactive rabbits did not develop resistance to challenging doses of virulent *T. pallidum*.

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Études immunologiques sur les antigènes tréponémiques cellulaires. I. Changements sérologiques et résistance à l'infection chez des lapins immunisés avec des acides nucléiques tréponémiques

SOMMAIRE

Le développement de *Treponema pallidum* avirulent *in vitro* est en relation directe avec la biosynthèse de l'acide nucléique. Des lapins recevant par voie sous-cutanée un mélange contenant un adjuvant ADN présentent des anticorps VDRL et FTA-ABS. Ces lapins séro-positifs n'ont pas montré des résistance vis-à-vis d'une inoculation ultérieure de *T. pallidum* virulent.